Absolute measurement centrifuge

Related applications and patents

This is a continuation-in-part of Application number 10/202,777 filed 24 July 2002.

The following patents and applications relate to the methods of light scattering for the measurement of molecular and particle mass and size.

P. J. Wyatt, U. S. Pat. No. 6,411,383 B1 (25 June, 2002) "Method for measuring the 2^{nd} virial coefficient."

S. Trainoff and P. J. Wyatt, Application No. 10/205,637 filed 24 July 2002 "Method for determining average solution properties of macromolecules by the injection method."

Background

Of all the devices that may be used for measuring the sizes of particles in the nanometer range, the disk and ultra centrifuges are among those most capable of providing high-resolution separations. Despite such resolution capability, the operation of such centrifuges is generally fraught with considerable ambiguities. Most of these problems are associated with uncertainties in the derived sizes of particles since such sizes are based entirely upon the arrival times of the separated particles at the detector. By using a mixture of the unknown sample particles with particles whose sizes are precisely known, these arrival times may be calibrated to some extent. Unfortunately, despite such calibrations, small variations in temperature and rotor speed, in addition to so-called streaming phenomena, often render such calibrations questionable. Another major difficulty relates to the need to know precisely the

density of the assumed homogeneous spherical particle structures and that of the fluid environment in which the separation is performed. Whenever a gradient is used, the explicit density variation should be known as well. Other problems associated with determining particle size by measuring times-of-arrival at the detector include deviations of Reynolds' number in excess of 0.5%, effects of sample dispersion due to Brownian motion resulting in the spreading out of the arrival times of identical particles, band broadening dependent on the speed of separation, establishing suitable gradients to prevent streaming, overloading sample concentration, range of particle sizes in the sample, problems with deconvolution analyses, *etc.* Virtually all these difficulties are associated with one basic shortcoming of these devices: centrifugal separation is not an absolute measurement method for most classes of particles. In other words, with the exception of a theoretical arrival time for homogeneous spheres at the detector, once a set of particles has arrived, their size cannot be measured directly. Of course, if the particles are not homogeneous spheres, *i. e.* of unknown structure, even the best of prior calibration procedures can result in great uncertainties in interpretation.

Centrifugal separation would appear ideally suited for the subsequent application of a multiangle light scattering, or MALS, analysis were it not for the inaccessibility of the samples. Thus, using cross flow field flow fractionation as described by Wyatt, for example, in his 1998 article "Submicrometer particle sizing by multiangle light scattering following fractionation," that appeared in *J. Colloid and Interface Science* volume **197,** pages 9—20, multiangle light scattering analyses of the eluant samples following separation produces detailed and accurate size and distribution information. The concept has been applied also to the analyses of samples separated by other methods including size exclusion chromatography

and capillary hydrodynamic fractionation, to name a few. A centrifugal device with an accessible eluting sample following separation was developed by J. Calvin Giddings and is referred to as sedimentation field flow fractionation, or SdFFF for short. This method, described, for example by Giddings in his 1993 paper in volume **260** of *Science* at pages 1456 *et seq.*, required an elaborate set of slip rings and capillaries. Other types of FFF separation techniques are also discussed in Giddings' paper. Combined with MALS, the analysis of eluting samples permitted the accurate characterization of each eluting fraction of particles independent of diffusion effects. Nevertheless, the SdFFF device had neither the resolution nor dynamic range of the more conventional centrifugal separation devices and was prone to leaks within a short time of installing new seals.

Results derived from the more conventional disk centrifuge and analytical centrifuge devices are based on the optical examination of small regions within the sample volume being subjected to centrifugal forces. Remote light sources, *i. e.* stationary relative to the spinning samples, are synchronized to the motion of the sample through the incident light beam to yield some measure of particle presence in the particular region being "interrogated." These may include absorption and forward scattering measurements as well as fluorescence for some devices. From such measurements, further attempts are usually made to derive a size distribution of the particle sample by interpreting the scattering and/or obscuration of the transmitted light beam at the detector in terms of Lorenz-Mie scattering theory, *i. e.* assuming the particles are homogeneous spheres. The forward-scattered light intensity is assumed to arise because such spheres of a known radius, a, have entered the incident light beam.

However, such "known" size was extracted from the time of arrival of the particles based on the relation

$$D \approx \frac{\sqrt{18\eta \ln(R/R_0)}}{\omega\left(\rho_p - \rho_f\right)^{\frac{1}{2}} t^{\frac{1}{2}}}, \qquad (1)$$

where $D = 2a$ is the particle diameter, ω is the angular velocity of the rotor, R_0 is the radius at which the sample particles were injected at time $t = 0$, R is the radius at which they are detected, η is the fluid viscosity, and ρ_p and ρ_f are the particle and fluid specific gravities, respectively. Possible sources of error in the terms of Eq. (1).can be significant. Most importantly, Eq. (1) only applies strictly for the case of homogeneous spherical structures.

Perhaps the greatest source of error in deriving particle size from Eq. (1) occurs when the particle density is close to that of the medium which is the case, for example, for proteins and a variety of particles produced by emulsion polymerization. When ρ_p and ρ_f are very close, slight errors in ρ_p can result in significant errors in the derived particle diameter, D. In addition, of course, Eq. (1) applies only to spherical particles. For non-spherical particles, the hydrodynamic radius, r_h, derived is just that of an equivalent sphere. It is an objective of this invention to provide a means by which the hydrodynamic radius of a particle passing through the detection beam may be determined far more accurately and without reference to a known particle standard, often used for centrifuge calibration. In addition to a measurement of the hydrodynamic radius, a particularly useful objective of this invention is the measurement of the so-called mean square radius. Knowledge of both of these radii often permits the derivation of the particle structure as well.

It is a further objective of this invention to provide an absolute measure of the radius of a spherical particle in the range of about 10 through 1000nm without calibration. An additional objective of this invention is to permit the accurate derivation of the particle size distributions of particles separated by centrifugal means even in the presence of significant diffusion caused by Brownian motion. Another objective of this invention is to circumvent, whenever possible, distortions in derived size distributions caused by other effects that tend to broaden the separated particle bands that appear at the detector such as systematic variations in rotor speed, changes in fluid temperature and viscosity, *etc.* Still another objective of this invention is the ability to measure sizes and size distributions for a broad range of inhomogeneous particles whose individual density variations may not be known *a priori*. Because some implementations of the disk and ultracentrifuges purport to be able to measure the concentration of very small particles directly, another objective of this invention is to be able to measure the molar mass of certain classes of molecules separated by centrifugal means. The success of the present invention to achieve these objectives depends critically upon the ability to integrate a MALS detection system into a centrifugal separation device and to use the existing features of centrifugal devices to permit more accurate analyses of the measured samples.

Brief descriptions of the drawings

Figure 1 shows the general structure of a disk centrifuge with transparent walls.

Figure 2 shows an end-on view of a disk centrifuge indicating the liquid meniscus and the radially increasing sample band.

Figure 3 shows a modified disk centrifuge with one wall containing an optical berm.

Figure 4 shows a detector configuration based on the berm structure of Fig. 3 permitting the measurement of light scattered by the sample over a range of angles.

Figure 5 shows a detector configuration based on the flat walled structure of Fig. 2

Figure 6 corresponds to an alternate form of a disk centrifuge wherein the samples are contained in discrete sample cells or cuvettes.

Figure 7 is a schematic layout of the optical system for the Beckman Optima XL-A Analytical Centrifuge.

Figure 8 shows a preferred embodiment of a sample cuvette incorporating a cylindrical lens for use in an ultracentrifuge incorporating discrete sample cuvettes.

Figure 9 shows a preferred embodiment of the detection system for the Beckman Analytical Centrifuge.

Figure 10 shows the mounting of laser and UV/visible light sources in juxtaposition to be used to provide sequential illumination of the samples.

Summary of the invention

The present invention permits the sizing of particles separated by sedimentation methods, such as a disk or analytical centrifuge, without requiring the use of standards for calibration. Because of this capability, problems with the separation mechanisms themselves are readily detected. Most centrifugation separation and subsequent sizing methods are based upon measuring the intensity of a highly collimated beam of light that has passed through a sample

undergoing sedimentation separation. The transmitted light beam provides a measure of the sample absorption as well as forward scattering. By correcting for the contributions of forward scattering, assuming that the particles are homogeneous spheres and using Lorenz-Mie scattering theory, and applying a size/time relation such as shown in Eq. (1), the particles' effective size is derived. Most devices that use such beam geometries, such as Koehler, *et al.* in their U. S. patent number 4,311,039, need to collimate the light source and detector so that the detected light corresponds to that interacting with the volume containing the small fraction of particles being illuminated.

The present invention modifies the detection of light passing through the sample by adding optical elements, masks, and an array of detectors thus permitting measurement of the light scattered by the sample over a range of angles. This multiangle light scattering detection device permits the determination of the scattering particles' mean square radius that, for a wide range of particle shapes, may be expressed as an effective particle size. Multiangle light scattering is often referred to by the acronym MALS. From such measurement of each fraction passing through the incident light beam, a size distribution may be derived that is absolute and independent of the time of arrival of the sample at the detector. For many types of particles in the submicrometer size range, these measurements are virtually independent of both particle density and refractive index.

The inventive concepts disclosed further have immediate application to other devices utilizing centrifugal forces for separation. Whereas many practitioners of such separation processes have been reliant upon the use of calibration standards as well as having to make

the often overwhelmingly precise measurements of the physical parameters of the samples and fluids involved, with the present invention the entire measurement process and subsequent interpretation will be simplified significantly.

Detailed description of the invention

The typical rotor and sample containing elements of a disk centrifuge are shown in Fig. 1. These include the transparent side plates **1** and **2** forming the sides of a fluid-containing sample chamber therebetween and a central rotor hub **3** attached to one of the plates. A fine collimated light source is shown at **4** and detector cell at **5**. Samples are introduced generally through the center opening **6** of **1** via channel **7**. Such and similar structures have been disclosed, for example, in the U. S. Patents 4,311,039 by Koehler *et al.* and 5,786,898 by Fitzpatrick. Figure 2 shows an end-on view of the sample chamber during operation. The particle sample is inserted through the opening **6** concentric with the axis of rotation **8** through the rotor **3** so that it begins its separation once in contact with the fluid meniscus **9**. Because of centrifugal forces, the sample migrates outwardly and eventually reaches the chamber's outermost boundary **10** where it remains until the chamber is cleaned. As each fraction of the sample reaches the detection region **11**, it passes through the incident light beam **12** from source **4** that is monitored at detector **5**. The light beam is generally well collimated and often monochromatic, for example, when the source a laser. On the other hand, it may be generated by a monochrometer providing a beam of controlled variable wavelength. Such monochrometers are often provided as part of the apparatus of analytical centrifuges. For certain classes of particles, a well-collimated light beam from a UV source is preferable.

The use of such centrifuges for the determination of particle size and particle size distributions based on the type of attenuated transmitted light measurements described above, is generally referred to as the "photosedimentation method". Because the associated separation theory refers specifically to particles of spherical shape, the versatility of the method becomes extremely limited and eventually requires for most measurements the introduction of "calibrated" standards. The departures from both theory and interpretation of arbitrarily shaped particles are rarely discussed in the literature or the patents based upon such measurements.

Although Eq. (1) is the form generally presented to show the relation between particle diameter D and arrival time t, it is instructive to examine its basis. As the chamber rotates at an angular velocity ω, a particle of mass m is forced outwardly by the centrifugal force $mR\omega^2$, where R is the distance from the axis of rotation **3**. However, if the particle density is ρ_p, the fluid density is ρ_f, and the particle occupies a volume V, then the centrifugal force becomes $(\rho_p - \rho_f)VR\omega^2$. For the case of a spherical particle of radius a, the centrifugal force is simply $4\pi a^3(\rho_p - \rho_f)R\omega^2/3$. Opposing the radial motion is the so-called Stokes' force which, for a sphere, is just $6\pi\eta a\, dR/dt$ where a is the radius of the sphere and η the viscosity of the fluid. Note that this latter formula applies only to a sphere and, therefore, the only result derivable in closed form. The net radial force on a spherical particle, therefore, is just the difference of the two forces, *i. e.*

$$m\ddot{R} = 4\pi\left(\rho_p - \rho_f\right)R\omega^2 a^3/3 - 6\pi\eta a\dot{R} \qquad (2)$$

or, since $m = 4\pi a^3(\rho_p - \rho_f)/3$,

$$\ddot{R} = R\omega^2 - 9\eta\dot{R}/\left[2a^2(\rho_p - \rho_f)\right]. \tag{3}$$

Thus

$$\ddot{R} + b\dot{R} - \omega^2 R = 0. \tag{4}$$

Equation (4) is readily solved in the general form

$$R = C_1 \exp(\alpha_1 t) + C_2 \exp(\alpha_2 t), \tag{5}$$

where $\alpha_{1,2} = \dfrac{-b \pm \sqrt{b^2 + 4\omega^2}}{2} = -b/2 \pm \sqrt{(b/2)^2 + \omega^2}$,

and $b = 9\eta/[2a^2(\rho_p - \rho_f)]$. At $t = 0$, $R = R_0$, the radius at which the sample is injected. Thus $C_1 + C_2 = R_0$. Also $\dot{R} = 0$ at $t = 0$, so $C_1\alpha_1 + C_2\alpha_2 = 0$. Combining these initial conditions to determine the coefficients C_1 and C_2 results in the final expression for the position, R(t), of the spherical particle as a function of time:

$$R(t) = R_0 \exp(-bt/2)\left[\cosh\frac{b}{2}\sqrt{1 + (2\omega/b)^2}\,t + \sinh\frac{b}{2}\sqrt{1 + (2\omega/b)^2}\,t\right]. \tag{6}$$

For typical separations for which ω is of the order of $2\pi 10^4$, where the fluid is water, the sphere is of diameter 100nm, and the density difference between the particle and fluid is of the order of 5×10^{-2}, the quantity $2\omega/b \ll 1$. Equation (6) then becomes simply

$$R = R_0 \exp(\omega^2 t/b). \tag{7}$$

Solving Eq. (7) for D yields Eq.(1) immediately. Note that all of these results apply to spheres only and are affected considerably by even slight differences between the actual particle and fluid densities and those measured. For the case of inhomogeneous particles,

even of spherically symmetric shape, the average particle densities well may vary with particle size making the interpretation of Eq. (1) even more uncertain.

The general chamber structure of the disk centrifuge per, for example, the device previously referenced by Koehler, *et al.* will result in a cylinder of fluid confined between two plates. Particles confined in such rotating fluids will be subject to both centrifugal and Coriolis forces. The Coriolis force, of magnitude $2\omega\dot{R}$ causes a motion in the direction of rotation. Since the ratio of the Coriolis force to the centrifugal force, $\omega^2 R$, is $2\omega/b$, we see that it is generally negligible.

Hoffman discloses an application of a disk centrifuge manufactured by Horiba, Ltd. of Kyoto, Japan, in his U. S. Patent number 4,871,248. The Horiba disk centrifuge, *e. g.* their CAPA 500, makes use of cuvettes mounted within the disk structure that rotates in a horizontal plane. These small cuvettes restrict any motion in the direction of rotation and eliminate thereby any Coriolis effects, no matter how great the angular velocity of the system. Thus samples are placed into the cuvettes as uniform dispersions that separate into specific populations during the spinning of the disk.

Light scattering is perhaps the best-known means for measuring the size of particles. Measurement of the scattered light intensity, as a function of scattering angle can be used to deduce such size for many diverse types of particles. For the case of a spherical particle, measurement of such multiangle light scattering patterns may be used to derive both sphere diameter and refractive index. Phillips, Wyatt and Berkman have demonstrated this, for

example, in their 1970 paper appearing in the Journal of Colloid and Interface Science, volume 34, pages 159 to 162. The structure of particles exhibiting spherically symmetry may also be deduced in some cases by measurement of their light scattering properties. Examples of the application of MALS measurements to the determination of such structures may be found in the following papers, for example:

"Cell Wall Thickness, Size Distribution, Refractive Index Ratio, and Dry Weight Content of Living Bacteria (*Staphylococcus aureus*)," Nature **226**, 277 (1970).

"Dielectric Structure of Spores from Differential Light Scattering," *Spores V*, American Society for Microbiology, (1971).

"Structure of Single Bacteria from Light Scattering," with D.T. Phillips, J. Theor. Biol. **37**, 493 (1972).

"Some Chemical, Physical and Optical Properties of Fly Ash Particles," Applied Optics **14**, 975 (1980).

For very small particles whose radii are less than about 10 nm, their size cannot be determined by MALS for visible incident light. This limitation is due entirely to lack of resolution at the wavelengths used for the measurements. Thus MALS cannot be used to derive the size of proteins, for example, whose size is typically of the order of a few nanometers. However, since various centrifuges, such as the analytical ultracentrifuge of Beckman Instruments, are equipped with advanced absorption optical systems, they are capable in principle of measuring the absolute concentration at any point in the sample. With the MALS capability of the present invention, it is possible to derive protein molar masses

directly by combining the light scattering data with the concentration results. Historically, it must be mentioned that measurements with the ultracentrifuge have been combined with other measurements such as light scattering, quasielastic light scattering, and viscometry in order to understand better the traditional ultracentrifuge results when the particles or proteins of interest are not spheres.

When the refractive index of particles subject to MALS measurements is close to the refractive index of the suspending fluid, there are several simplifications in the theoretical interpretation of the MALS measurements that permit the determination of the so-called rms radius of the scattering particles. For many simple structures such as spheres, rods, disks, ellipsoids, etc., the rms radius may be related directly to more characteristic size parameters such as radius or length. With some *a priori* knowledge of the particles' shape, the present invention permits, for many classes of particles, the determination of their size even though they are not spheres. In addition, for a reasonably wide range of refractive indices and corresponding densities, the sizes of even spherical particles may be determined accurately from their measurement in a centrifugal device incorporating the key features of the present invention. As clearly evident from Eq. (1), slight errors in the determination of particle density can result in large sizing errors using devices based on centrifugal separations. The density of simple polystyrene spheres relative to water is only of the order of $5x10^{-2}$ and obviously any errors in this value will have a major effect on the determination of the corresponding particle size based on Eq. (1). The present invention eliminates this dependency to a great extent.

In order to make MALS measurements from a sample undergoing centrifugal separation according to the methods taught by the present invention, special optics and detector capabilities must be incorporated into the centrifugal devices. Figure 3a shows a preferred embodiment of an element of the invention. A transparent berm **12** has been integrated into the outer transparent plate of a disk centrifuge. A cross section of the plate with the integrated cylindrical berm is shown in Fig. 3b. In its preferred embodiment, the radius of curvature of the berm will be centered at the illuminated sample.

Figure 4 shows a finely collimated light beam **13** from light beam source **4** passing through the berm structure **12** and exiting at **14** before entering a transmitted light detector **15**. In the preferred configuration of the optical berm, the beam **16** exits normally through a flattened anti-reflection coated surface **14**. Also shown is a set of highly collimated detectors **17** each receiving light scattered by the sample region **18** into unique angular directions **19** with respect to the emerging beam **16**. Note that the light scattered from the sample region **18** leaves the optical berm normal to its surface. The detectors **17** are collimated so as to accept light scattered from the sample region **18** within a small solid angle. Because the separations of particles generally span a small range of centrifugal radial distances **20**, it is essential that the detector collimation provide a high degree of sample/solute resolution with respect to the radial direction. This is achieved by collimation that provides for a highly localized coincident field of view at each distinct detector. Adding optical lenses in front of the detectors would further restrict the depth of field and, thereby, the contributing scattering volume detected. The berm structure **12**, of course, may be eliminated completely resulting in the emergence of scattered light through the flat outer plate surface as shown in Fig. 5. The

specific masking and detector orientation required to detect light scattered from the same highly restricted scattering region would add some complexity to the apparatus. Most importantly, the efficiency of collecting scattered light would be somewhat reduced and internal reflections at the interfaces increased somewhat. Some additional masking of the detectors might be needed also to minimize the passage of stray light into the detectors. In the preferred embodiment of this invention, the light would be from a laser source and would be plane polarized perpendicular to the plane containing the detectors **17** shown in both Figs. 4 and 5. Although such co-planar detectors are sufficient to provide the characterizing data that would form the basis for subsequent analysis of the molecules/particles being measured, for some classes of measurements or because of geometrical limitations, detectors outside of such a preferred plane may be employed. Such detectors would be classified by both a scattering angle and an azimuthal angle with respect to a defined plane.

The structure shown in Fig. 4 and Fig. 5 for the detection of scattered light from a disk centrifuge sample is quite different from the conventional transmitted beam detection shown in Fig. 1. Most importantly, the particles in the illuminated region **18** are detected by the light they scatter rather than the more conventional absorption means achieved by monitoring the transmitted beam. By integrating into the disk structure a transparent berm whose cross section provides an optical surface normal to the scattered light, it becomes possible to measure the scattered light intensity over a greater range of angles than would otherwise be the case were the surface planar. For the berm structure, this range could be between 10 degrees and 80 degrees. Adding anti-reflection coating over the surfaces through which the scattered light passes will enhance the transmission characteristics of the optical berm

structure further. For the case of a flat surface as shown in Fig. 5, this range is substantially reduced. Thus while the flat surface simplifies significantly the construction of the sample chamber, it results in a far greater reduction in the accessible scattering angle range. The maximum scattering angle accessible with a flat surface is in the range of 40° to 45° which is far less than the almost 80°accesible using the berm structure. For both flat and berm surfaces, the forward transmitted beam **16** is measured at detector **15** which may serve also as a light trap, preventing, thereby, the presence of stray light that might be scattered by adjacent structures into the MALS detectors **17**. The trap incorporated therein could consist of a Rayleigh horn or even a mirror or prism structure that would remove the incident beam and send it in a region where any light scattered from such deviated beam would not be detectable by any of the collimated detectors **17**. The trap could be comprised also of an optically dense and non-reflecting medium such as anti-reflection coated black glass. However, for measurements of the intensity of the transmitted beam **16**, which for many of the implementations of the photosedimentation method is required to calculate the associated particle/molecular concentration, a combination of a beam intensity detector and a beam trap will be required. Because of the significant refractions at the flat surface of scattered light shown in Fig. 5, the angular position of the detectors relative to the incident light beam is shown by the lines **19a**. The actual scattering angles are indicated by the angles **19**.

Once again, although the berm structure is the preferred embodiment of the sample chamber, for very small particles/molecules whose scattering is close to isotropic, the flat surface and its reduced range of accessible scattering angles could be used without too great a degradation of the particle/molecule size derived therefrom.

Figure 6 shows the basic elements of the Horiba disk centrifuge discussed earlier. Two cuvettes, containing the sample and reference fluids respectively, are placed at **21** and **22** within the horizontal disk **23**. The light source and detector is similar to that used with the Koehler *et al.* device discussed earlier though since the cells or cuvettes, and associated sample, occupy only a small part of the circumference **24** traced out by the light source-produced beam as the disk rotates. The light source **4** and/or detector **5** may be pulsed or modulated so that they are turned on only during the period the sample or reference cuvettes are in the beam. High resolution of a sedimenting sample requires that the light beam diameter be as small as practical. For the Horiba device, the beam diameter is an order of magnitude greater at 2 to 3 mm than that of currently available laser sources preferred for the present invention.

The analytical ultracentrifuge, of the type sold by Beckman Instruments, Inc., represents a device quite distinct from the disk centrifuges discussed earlier. Because they can achieve far greater speeds, up to 60,000 revolutions per minute, they are able to separate far smaller particles. Indeed, one of the main applications of such systems is for the study of proteins. Such molecules are distinguished by their small size, rarely exceeding a few nanometers, and their associated greater diffusion coefficients. The determination of molecular weight, shapes, sizes, distributions and purity may, in principle, be derived directly from careful measurements of various features of a sedimenting sample. Such measurements include the need to observe and detect sharp boundary regions in the separating samples. The analytical ultracentrifuge relations used to derive molar mass, for example, are quite different from Eq.

(1) used to derive the diameter of the separated particles. Rather than make assumptions concerning the molecule's shape and assuming that Stokes' law may describe the viscous drag force, the frictional force is assumed to be of the form $F_f = -fu = -f\,dR/dt = -f\dot{R}$, where f is the so-called frictional coefficient which depends on the particle's size and shape. The particle mass m is expressed in terms of its associated molar mass M by dividing by Avogadro's number N_a, *i. e.* $m = M/N_a$. Thus Eq. (2) is generalized to the form

$$\frac{M}{N_a}\omega^2 R - \frac{M}{N_a}\bar{v}\rho_f\omega^2 R - f\dot{R} \approx 0, \tag{8}$$

where a steady state has been assumed to exist, *i. e.* $\ddot{R} \approx 0$. The second term in Eq. (8) corresponds to the contribution of buoyancy, where ρ_f is the density of the solvent and $\bar{v}$ is the volume in g/mL displaced by each gram of the molecule. Combining the terms of Eq. (8) results in

$$s = \frac{\dot{R}}{\omega^2 R} \quad , \tag{9}$$

where $s = \frac{M}{N_a}\left[1 - \bar{v}\rho_f\right]$ is the sedimentation coefficient. For relatively sharp and symmetrical sedimenting boundaries, the sedimentation coefficient is obtained by integrating $\frac{\dot{R}}{\omega^2 R}$ to yield

$$\ln(R/R_m) = s\omega^2 t, \tag{10}$$

where R is the boundary midpoint and R_m is the meniscus position. Note the similarity of Eq. (10) and the corresponding result for the disk centrifuge of Eq. (7). A plot of $\ln(R)$ versus t yields a straight line of slope $\omega^2 s$ from which s may be calculated. Next, measurement of the

rate of boundary spreading can be used to calculate the diffusion coefficient D that will depend on the effective size of the diffusing molecules through the corresponding frictional coefficient f. Thus

$$D = \frac{\Re T}{N_a f}. \tag{11}$$

The absolute temperature is T and $\Re$ is the gas constant. Taking the ratio of the sedimentation to the diffusion coefficient using Eq. (9) and (11) gives the molar mass

$$M = \frac{s^0 \Re T}{D^0 (1 - \bar{\nu}\rho)}. \tag{12}$$

The superscripts indicate that the calculated diffusion and sedimentation coefficients have been extrapolated to zero solute concentration. Each is generally calculated from measurements made using solutes at different concentrations.

The derivation of molar mass results based on Eq. (12) involves both a great amount of time and extensive calculations. The determination of the partial specific volume $\bar{\nu}$ itself is generally no trivial matter. Indeed, the determination of molar mass, though absolute, is most difficult to measure by analytical ultracentrifuge. The preferred method by which molar mass is determined using the analytical ultracentrifuge is by means of the technique of sedimentation equilibrium. A small volume of an initially uniform solution is centrifuged at lower speeds than generally required to obtain the molar mass by means of the sedimentation velocity method of Eq. (12). This results in a concentration gradient building up from the bottom of the cuvette. The molecular diffusion increases with increasing concentration so that there are two counter flows at each concentration: a radial flow caused by the centrifugal force and an opposite flow due to diffusion. Eventually an equilibrium concentration

distribution is achieved where the concentration of the molecular species varies exponentially with R^2. For a monodisperse non-associating molecular solute, the molar mass may be shown to be

$$M = \frac{2\Re T}{(1-\bar{v}\rho)\omega^2}\frac{d(\ln c)}{d(R^2)}. \qquad (13)$$

Thus a plot of ln c versus R^2 yields a slope directly proportional to the molar mass, M. Alternatively, by fitting the data of c versus R^2 to an exponential using a least squares' fit, one should be able to derive an estimate of $M(1-\bar{v}\rho)$ directly.

Despite the great difficulties associated with finding molar masses directly using the analytical ultracentrifuge, the power of the instrument to separate such small molecules while at the same time being able study a wide range of other phenomena such as heterogeneity, association reactions, and a variety of thermodynamic properties make the analytical centrifuge a most useful analytical tool. Because the Beckman device measures concentration directly, were the instrument combined with the elements of the present invention, its utility would be extended significantly. Thus molar masses could be calculated directly by combining concentration measurements with an absolute measurement of light scattered by the samples being studied. Once molar masses were so easily obtained, more accurate values of both sedimentation and diffusion constants could be derived almost effortlessly. The means by which the preferred embodiment of the present invention may be applied to the analytical ultracentrifuge will now be discussed.

Figure 7a presents a schematic of the optical system of the Beckman analytical ultracentrifuge. The sample holding rotor **25** rotates about shaft **3** within an evacuated

chamber. Similar to the Horiba structure of Fig. 6, the rotor contains sample and reference cuvettes. However, two pairs are included at diametrically opposite locations **26** and **27**. Figure 7b shows a top view of one of these locations, for example **26**, containing sample **28** and reference **29** cuvettes. By this means, two distinct samples may be processed during each experiment. Note that each cuvette is constructed with a side boundary lying along a radius at a slight angle to the other side that lies along the principal diameter of the rotor. This structure helps reduce internal sample streaming during separation. A Xenon flash lamp source **30** is shown together with a steering diffraction grating **31** and incident light monitor **32** that receives a small signal proportional to the incident intensity by means of a beam splitter **33**. The focused beam **34** passes sequentially through the sample and reference cells when cell-containing regions **26** or **27** are in the beam. In general, the incident light source is flashed so that the beam is on only during its passage through the sample or reference cells. The diffraction grating permits also the selection of the wavelength of the incident beam over the range of possibilities associated with the light source; in this case, a Xenon lamp. Generally wavelengths in the near ultraviolet are selected as a great many studies with such apparatus involve proteinaceous materials that absorb strongly in the UV. An optical imaging system **35** collects light transmitted through the sample within **26** or **27** from a small radial region within the sedimenting sample. The image of the mask **36** at the illuminated sample defines this narrow field of view. A photodetector such as a photomultiplier tube **37** detects this transmitted light, though other detection systems may be employed.

The structure comprised of the beam **34**, imaging system **35**, and photodetector **37** are controlled to move in the radial direction as a unit permitting, thereby, the sample to be

measured at different radial distances. For the disk centrifuges, on the other hand, the beam/detector pair is set at a single radial distance throughout the entire measurement. Depending upon the types of particles/molecules to be separated, the radial scans are repeated many times to yield a sedimentation profile as a function of time. In the preferred embodiment of this invention, when elastically scattered light measurements are implemented, the preferable light sources will be lasers. The optical systems can accommodate both UV flashed sources as well as lasers so that both concentration and so-called static light scattering measurements may be made almost simultaneously.

Unlike the disk centrifuge of Figs.1 through 5, the analytical ultracentrifuge and the cuvette based disk centrifuge cannot benefit from the circular berm lens structure disclosed earlier. In their preferred embodiment, the individual cuvettes *per se* must incorporate the required cylindrical lens structure. They may be of the simpler flat surface construction, as well; however the range of accessible scattering angles is minimized because of the surface refractions. In addition as the refractions approach the critical angle, the intensity of the transmitted scattered light becomes greatly attenuated. Nevertheless, for smaller particles/molecules, the reduced range of scattering angles accessible with a flat exit surface may be sufficient to derive their corresponding sizes. For very small particles/molecules Figure8 presents a section of a cuvette that could be used in the preferred embodiment of the present invention described in the parent case serial 10/202,777. Instead of having flat sides through which the light beam will pass, the flat surface through which the beam exits has been modified to provide a cylindrical structure **38** whose radius of curvature, in the preferred embodiment, is centered at the illuminated sample. For the ultracentrifuge, the

beam also may be scanned radially along the length of the cuvette in the direction of increasing radius **39**; while for the disk centrifuge incorporating such cuvettes, the beam will be restricted to a fixed position. This cylindrical surface of the preferred embodiment may have a flattened apex **40** corresponding to the similar structure for the berm. The entire region may be antireflection coated, as well. Once again, the standard flat cuvette may be used to make measurements over a restricted range of scattering angles.

Figure 9 shows an instantaneous cross sectional view of the preferred embodiment for an analytical ultracentrifuge. Also shown are the imaging system **35** and the photomultiplier detector **37** of the conventional structure of Fig. 6a. In the region between this imaging system **35** and the rotor housing **25** are a set of collimated scattered light detectors **41** similar to the detectors **17** of Fig. 4. These detectors, as well as those shown in Fig.4, generally lie in a plane and intercept scattered light from a small illuminated volume **38** within the cuvette/cell **28**. They move with the other elements of the imaging system and collect scattered light throughout the radial scanning transverse to the plane of the figure. Thus the sample particle mass and size distribution profiles throughout the scanned cuvettes may be determined from the scattering measurements and recorded by the inventive system described. The light beam **34** is therefore always perpendicular to the imaging system **35** and the photomultiplier tube **37** to which are attached the scattered light detectors **17**. Thus all detection, light sources, and optical elements are on a fixed platform relative to the rotor **25**. The platform may move radially, permitting, thereby, the radial scanning capability of the analytical ultra centrifuge. The ability to make radial scans during operation of conventional disc centrifuges is a feature not seen with such devices, but is relatively easy to implement.

When the reference cuvette **29** arrives at the position previously occupied by the sample cuvette **28**, light scattered by its solution will be collected in a similar manner.

The light source producing the beam incident upon the sample and reference cuvettes can originate from a laser source or, alternatively, from a variable UV/visible light source such as commonly used in the analytical ultracentrifuge. On the other hand, both variable and laser sources may be activated sequentially or simultaneously. In Fig. 10 a laser source **42** used to produce a light beam to interrogate the sample is shown mounted in juxtaposition to the steering diffraction grating **31.** Both lie at the same radial distance, but displaced in rotation angle. The UV source beam **34** from the Xenon flash lamp **30** will strike the cuvette after the incident the laser beam **43** has passed through it as the cuvette rotates counterclockwise through the same radial position with respect to the cuvette-contained separating sample **28.** The corresponding transmitted UV/visible light beam **34** intensity, used to calculate the sample absorption, and the light scattered from laser beam **43** into detectors **17**, are combined to calculate size and mass. The signals UV and laser beam interactions with the sample are collected sequentially at the same radial position of the sample. Note that the UV/ visible light source itself has an associated set of scattered light detectors **41** to detect scattered light from the sample. The UV/ visible light source may be replaced equivalently by any other light source or sources and used, for example, with a photodiode array detector.

Alternatively, the laser beam may be arranged to be collinear with the UV/ visible light source, or to replace it if the determination of the molar mass using concentration detection means is not required. A multiwavelength laser or light source may be selected as well, with

various filters chosen to select the transmitted beam wavelength. Still other filters may be selected and attached to the scattered light detectors to eliminate detection of specific scattered wavelengths. There are many other means for providing such beams, as would be obvious to those skilled in the art of optical design

The dual source geometry discussed above for the case of the analytical ultracentrifuge, whereby the sample is illuminated sequentially by the two different light sources as the sample containing region rotates past them, may be applied as well to the disk centrifuge geometries discussed earlier. This dual sequential illumination may be implemented with the structures of Figs. 2, 4, or 6. Indeed, multiple illumination sources may always be used for any of the centrifugal separation devices. Such multiple sources are not restricted to two.

If the laser and UV/ visible light sources were oriented so that they formed a single incident beam, then the scattered light detectors **17** would be fitted preferably with filters to remove UV/ visible light scattered by the solutions. Such filters would be preferably interference filters permitting only elastically scattered light at the laser wavelength to be detected. If inelastically scattered laser light is to be detected, such as caused by fluorescence, the corresponding detector filters would be selected accordingly. If any of the light sources employed is polarized, polarization sensitive analyzers may be attached to selected scattered light detectors to permit quantitative measurement of depolarization scattering effects.

It is clear from the discussions above that the key elements of this invention apply equally to various types of instrumentation using photometric means to monitor sedimentation

phenomena induced by an applied centrifugal force. The purpose of the invention is to permit the measurement of scattered light from regions of the sample being separated by such means. From such measurements made over a range of scattering angles, it becomes possible to derive particle size directly, irrespective of diffusion phenomena. For the case of sub-micrometer particles, means by which such scattered light measurements may be used to measure particle size and size distributions has been explained in such papers as: "Absolute Measurement of Diameter Distributions of Particles Using a Multiangle Light Scattering Photometer Coupled With Flow Field-Flow Fractionation," D.W. Shortt and D. Roessner, and P. J. Wyatt, Am. Lab. **28 17**, 21 (1996); and "Submicrometer particle sizing by multiangle light scattering following fractionation," P. J. Wyatt, *J. Colloid and Interface Science* **1979**, 9-20 (1998).

For the case of solvated molecules undergoing separation by centrifugal means, the weight average molar mass may be derived directly if the concentration of the molecules be known in addition to the differential refractive index increment, *dn/dc*. Details of such quantities may be found in the co-pending application by Wyatt and Trainoff referenced at the beginning of this specification as well as the reference by Wyatt discussed in the next paragraph. Most centrifugal separation devices, and certainly the analytical ultracentrifuge, use a light beam whose absorption by the solution may be used directly to calculate the concentration of the molecules present. Thus for the case of solvated molecules, a UV/ visible light source is often sufficient as it exists to produce absorption data sufficient to determine, from the sample's extinction coefficient, the molecular concentration. For larger particles, on the other hand, such absorption techniques rarely may be used to calculate the

particle concentration because of the role played by the particle scattering. In addition, the angular variation of such particle scattering is generally sufficient to calculate the effective particle size. Since the forward transmitted beam that passes directly through the sample is useful to determine the molecular concentration and, perhaps for some particles, the beam transmittance, the preferred embodiment of the invention would continue the use of such measurements.

The ability to measure molecular mass and size directly for samples undergoing centrifugal separation, and especially for proteins, is a particularly significant application of this invention as it potentially eliminates those elements most difficult to measure from conventional analytical ultracentrifugal analysis. Most important among such elements is the determination of the volume of solvent displaced by the molecule whose mass and size is to be determined. Once the concentration and light scattering response as a function of scattering angle of a particular molecular species are known, the molecular mass may be determined immediately following the methods described in detail by Wyatt in his 1993 Analytica Chimica Acta paper in volume 271, pages 1 *et seq.*, entitled "Light Scattering and the Absolute Characterization of Macromolecules." Once the mass of a separating species has been so-determined, the molecules' volume, for example, may be calculated explicitly from the sedimentation coefficient derived via Eq. (10). Such determinations have never been made directly in this manner. The implications of these direct determinations for the protein chemistry and related fields are of great importance.

Another key element of the invention relates to the modifications required of the exit surfaces of the sample-containing regions. As the beam leaves the sample, it should exit preferably normal to the transparent region in which the sample is restricted. Thus the normal surface through which the undeviated incident beam passes remains the same as that currently employed in such centrifugal separation devices. A major modification of the surface region is required so that the light scattered from the sample can be measured also over a reasonable range of angles. As has been mentioned previously, the invention is intended to permit measurement of light scattered over a range of scattering angles from a small volume within the illuminated sample. Thus scattered light leaves the sample-containing surface normal thereto in its passage to a set of detectors placed preferably at equidistant radial positions from the small scattering volume. The collimation of these detectors defines the field of view, *i. e.* the transverse dimension of the scattering volume. The optical surface itself, generally forming a cylindrical lens whose apex has been flattened as described previously, would have its radius of curvature centered at the illuminated sample volume. A flat surface may be employed if the range of accessible scattering angles may be restricted.

As will be evident to those skilled in the arts of light scattering, there are many obvious variations of the modified centrifugal separators I have invented and described that do not depart from the fundamental elements that I have listed for their practice; all such variations are but obvious implementations of my invention described hereinbefore and are included by reference to my claims, which follow.